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Effects of Bioconjugation with Novel Phospholipid Polymers on Enzymatic Activity and Stability

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ABSTRACT

Various compositions of poly(2-methacryloyloxyethyl phosphorylchorine (MPC)-co-n-butyl methacrylate (BMA)) (PMB-COOH) (Mw=1.0x10^4) with a carboxylic group on a terminal were synthesized by photoinduced living radical polymerization. These polymers were conjugated to an enzyme, papain. Then, the poly(MPC), PMPC-COOH, was conjugated to papain as a control. The modification degrees of the polymers were 16-19 %. The effects of BMA unit in the polymers on the enzymatic activity and stability were investigated. The remaining activity of papain conjugated with PMB-COOH decreased with an increase the BMA units and these values were small compared to papain conjugated with PMPC-COOH. However, PMB-COOH conjugated papains did not induce the change in secondary structure compared to that of the PMPC-COOH conjugated papain. The secondary structure were maintained for 28 days at 40 °C. Moreover, with increase in the composition of BMA units in the PMB-COOH, PMB-COOH conjugated papains maintained high enzymatic activity for 28 days at 40 °C. We concluded that the PMB could enhance effectively the stability of enzyme.

Key words: 2-methacryloyloxyethylphosphorylchorine, photoinduced living radical polymerization, enzymatic activity, stability, bioconjugation

1. INTRODUCTION

Bioconjugates between enzyme and synthetic polymer are excellent way to enhance enzymatic functions and stability[1]. In this step, the synthetic polymers do not show any adverse effect to enzymes after the conjugation. Also these polymers are required that the structure including the end groups is defined and the molecular weight distribution is narrow. One of the well-known polymers for bioconjugation is poly(ethylene glycol) (PEG), and its bioconjugation has been widely studied[2,3]. On the other hand, from the view point of polymer chemistry, since the chemical structure of PEG is quite simple, further chemical modification is difficult. We consider that an increase in the variation of the synthetic polymers for bioconjugation is important.

We newly focused on the use of vinyl polymers as conjugate polymer. Generally, the vinyl polymers, which have reactive end groups, have been synthesized by conventional free radical polymerization using a chain transfer agent[4-7]. In this method, it is hard to control both the molecular weight distribution and increase the molecular weight. To control the molecular weight distribution and the molecular weight, and to easily adapt a reactive end group, we used a photoinduced living radical polymerization method[8].

We have been recently developing bioconjugation with bioinspired water-soluble phospholipid polymer as a "nano-scaled molecular device". The phospholipid polymers composed of the MPC unit has an excellent biocompatibility as biomaterials[9]. In this study, novel MPC polymers with the reactive end group on one terminal was synthesized by using a photoinduced living radical polymerization. These MPC polymers were used for the conjugation with enzyme. In previous reports, poly(MPC) chain could stabilize the enzyme by control of the molecular weight of the poly(MPC) and modification degree to the enzyme[10,11]. Moreover, the enzymatic activity and stability of the enzyme conjugated with poly(MPC) was excellent compared to that of PEG conjugated the enzyme.

We consider that the molecular mobility of the enzyme is one of the dominant factors of the enzymatic stability. So polymers containing hydrophobic unit were conjugated to the enzyme. The molecular mobility of the enzyme is restrained by decreasing the exchange of water rate at the neighborhood of the hydrophobic unit.

In this study, various compositions of poly(MPC-co-BMA)s were synthesized and conjugated to the enzyme, papain. The effects of the amount of BMA for the enzymatic activity and stability of papain conjugated with poly(MPC-co-BMA) were investigated.

2. MATERIALS AND METHODS

2.1 Materials

MPC was synthesized by the method reported previously[12]. 4-(*N,N*-diethyldithiocarbamoylmethyl) benzoic acid (BDC) was synthesized by condensation between 4-chlromethyl benzoic acid and sodium *N,N*-diethyldithiocarbamate[10]. Papain was purchased from Nacalai Tesque (Kyoto, Japan). Other organic reagents and solvents were purified by the usual method.

2.2 Synthesis of PMPC and PMB with carboxyl group (PMPC-COOH and PMB-COOH)

The BDC(1) was dissolved in tetrahydrofuran (THF) (30 mL). MPC and BMA were dissolved in ethanol (150 mL). After purging with Ar gas, the solutions were poured into a glass tubing for mixing, and then the tubing was sealed. The polymerization was carried out by photoirradiation using a high-mercury lamp (Riko, Chiba, Japan) at room temperature for 3 hr. The mixture was poured into a large amount of chloroform to purify the PMPC-COOH and PMB-COOH(2) by precipitation. The obtained polymers were dried under reduced pressure for 2 days. The chemical structure of PMPC-COOH(2) and PMB-COOH(2) were confirmed by ^{1}H-NMR (500 MHz, JEOL α-500, Tokyo, Japan, in ethanol-d_6): PMPC-COOH, δ1.29 (d, 6H, -N-CH_3), 1.87-1.93 (d, 3H, α- CH_3), 3.21-3.30 (d, 9H, -N(CH_3)-), 3.60-3.73 (d, 4H, -CH_2-N-), 4.07-4.32 (d, 4H, -O-CH_2-CH_2-P), 7.89 (d, 6H, *benzyl*). The molecular weight of the PMPC-COOH(2) and PMB-COOH(2) was determined by gel permeation chromatography (JASCO, Tokyo, Japan; flow rate, 0.5 mL/min, detector, RI; eluent, 0.1 mol/L LiBr aqueous solution; Column, Shodex OHpak SB-803 (HQ) with well-defined PEO as reference samples (PEO standards, Tohso, Tokyo, Japan).

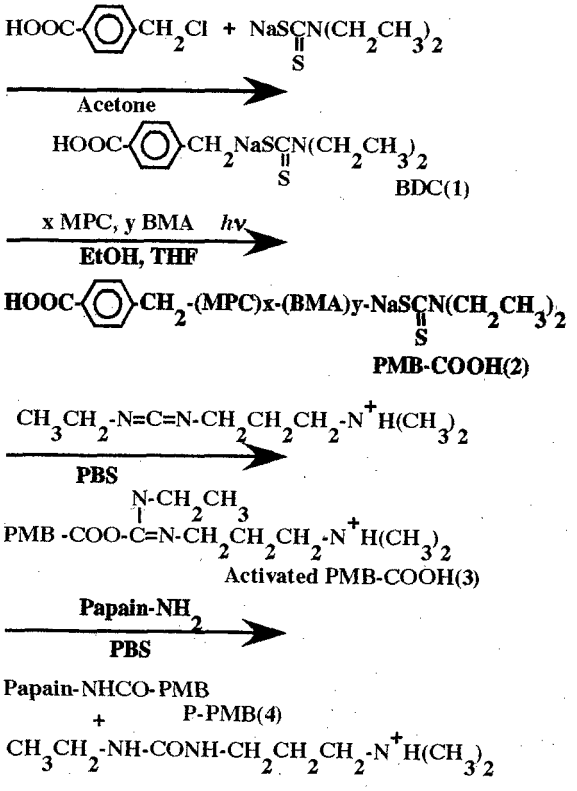

Fig. 1 Scheme for synthesis of PMB-conjugated papain

2.3 Conjugation of PMPC-COOH and PMB-COOH with papain

The carboxyl group at the terminal of PMPC-COOH(2) and PMB-COOH(2) was conjugated to the amino groups of papain after activating the carboxyl group. At first, 0.30 mol of PMPC-COOH(2) and PMB-COOH(2) were dissolved in 30 mL of 0.1 mol/L, pH 7.0 phosphate-buffered solution (PBS). Then 0.016 mmol of water-soluble carbodiimide (WSC) was added into these solutions and stirred for 2 hr. These solutions were added into 100 mg of papain/ 70 mL of 0.1 mol/L, pH 7.0 PBS and reacted at 4 °C for 24 hr. To remove by-product, unreacted papain and polymers, these solutions were dialyzed against the water at 4 °C for 24 hr. Moreover, these were purified by ultra filtration for 30 times. Finally, the papains conjugated with PMPC-COOH(2), P-PMPC(4) and PMB-COOH(2), P-PMB(4) were obtained by freeze-drying. The synthetic route of P-PMPC(4) and P-PMB(4) is schematically illustrated in Fig. 1.

The modification degree of the polymers against the total number of amino groups of papain was quantitatively determined by the sulfo-succinimidly-4-*O*-(4,4'-dimethoxytrityl) butyrate (sulfo-SDTB) method in order to the count residual amino groups of papain after conjugation.

The secondary structure of the native, P-PMPC(4) and P-PMB(4) was measured with a circular dichroism (CD) spectropolarimeter (J-720W, JASCO, Tokyo, Japan). The measurement was carried out at 40 °C and the concentration of papain was 3.10×10^{-8} mol/l.

2.4 Measurement of the enzymatic activity

The enzymatic activity of the native papain, P-PMPC and P-PMB was determined using benzoyl-L-arginine-ethylester (BAEE) as the substrate. BAEE was dissolved in 0.1 mol/L, pH 6.1 PBS and the native papain, P-PMPC and P-PMB/ 0.1 mol/L, pH 6.1 PBS which contains 1 mmol/L EDTA, then 5 mmol/L L-systeine was added. The reaction rate was determined by UV absorbance at 254 nm with a UV-vis photospectrometer (UV-650, JASCO, Tokyo, Japan). The reaction was carried out at 40 °C. The concentration of the native papain, P-PMPC and P-PMB was 18 unit/L.

3. RESULTS AND DISCUSSION

3.1 Characterization of PMPC-COOH and PMB-COOH

It is well known that polymers, which are synthesized by photoinduced living radical polymerization using an iniferter regulate the molecular weight and its distribution. In this study, we demonstrated the synthesis of new water-soluble polymers, which have both phosphorylcholine groups in the side chain and carboxyl group at one terminal of the polymer chain (PMPC-COOH and PMB-COOH) by photoinduced living radical polymerization using an iniferter, which had one carboxyl group.

The synthetic results of the PMPC-COOH and PMB-COOH are summarized in Table I. The molecular weight could be regulated about 1.0×10^4 based on feeding ratio of the monomers and iniferter. Moreover, the molecilar weight distribution of PMPC-COOH and PMB-COOH was narrow (Mw/Mn=1.35-1.45) when compared with a conventional free radical polymerization. The ^{1}H-NMR spectra data corresponded to the structure of the PMPC-COOH and PMB-COOH. Thus, these polymers were better adapted to conjugate with enzymes.

Table I Characterization of PMPC-COOH and PMB-COOH

Monomer /Iniferter	BMA mole fraction in feed	BMA mole fraction*	Mw**	Mw/Mn**
33.6	0	0	$1.1x10^4$	1.35
35.4	0.05	5	$1.0x10^4$	1.45
38.9	0.25	22	$1.0x10^4$	1.44
47.9	0.50	55	$1.2x10^4$	1.40

*Determined by ^{1}H-NMR
**Determined by gel permeation chromatography

3.2 Conjugation of polymers to papain

In Table II, the results of bioconjugation with the MPC polymers and papain are summarized. The papains conjugated with PMPC-COOH and PMB-COOH are described as P-PMPC and P-PMB, respectively. The modification degrees of P-PMPC, P-PMB5, P-PMB25 and P-PMB50 were 19, 19, 18 and 16 % versus amino groups of papain, respectively. Additionally, The remaining activities of P-PMB5, P-PMB25 and P-PMB50 were 35, 32 and 24 % respectively. These values were decreased with an increase in the BMA unit and were slightly small compared to the value of P-PMPC 41 % (Table II). But, these results were reasonable because the diffusivity of the substrate at the neighborhood of papain was restrained by increase of BMA units.

Table II Modification degree and remaining activity of papain conjugated with the MPC polymers

Code	Modification degree [%]	Remaining activity [%]
P-PMPC	19	41
P-PMB5	19	35
P-PMB25	18	32
P-PMB50	16	24

3.3 Stability of polymer-conjugated papain

Fig. 2 shows the change in the α-helix content of the native papain, P-PMPC and P-PMB stored at 40 °C. The α-helix content represents a parameter of the change in secondary structure of the enzyme. The α-helix content of the native papain was almost 25 %, but it decreased gradually and could not be detected after 14 days at 40 °C. This phenomenon indicates that the secondary structure of the native papain completely collapsed during these periods. On the other hand, the α-helix content of P-PMPC was about 20 % and it was almost maintained for 28 days. Moreover, the α-helix content of P-PMBs was about 23 % and it was maintained for 28 days. These results indicate that the change in the secondary structure did not occur for 28 days, even when it was stored at high temperature 40 °C. The PMPC chains did not show any adverse effects on the stability of papain. The stability of P-PMBs was increased. That is, the mobility of P-PMB was refrained by the introduction of hydrophobic circumstances.

Fig. 3 shows the enzymatic activity profile of the native papain and papain conjugated with the various MPC polymers when they were stored at 40 °C. The enzymatic activity of the native papain decreased with the storage period. This result corresponded to the change in the α-helix content of the enzyme with time. The enzymatic activity of P-PMPC was maintained at about 50 % of the initial enzymatic activity. This is inhibition of self-digestion and structure change in papain by conjugation with the PMPC chains. But, with increase in the composition of BMA units in PMB, the P-PMB bioconjugates maintained high enzymatic activity for 28 days. These results indicate that the stability of P-PMB was increased by refraining the mobility of P-PMB with an increase in the BMA units in polymers.

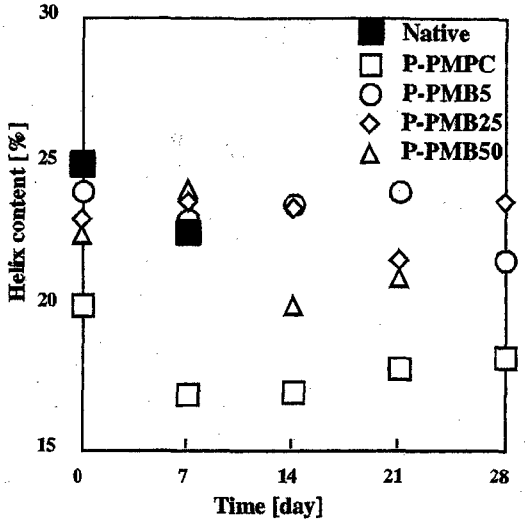

Fig. 2 Time dependence of α-helix content of native (■), P-PMPC (□), P-PMB5 (○), P-PMB25 (◇) and P-PNM50 (△) stored at 40 °C.

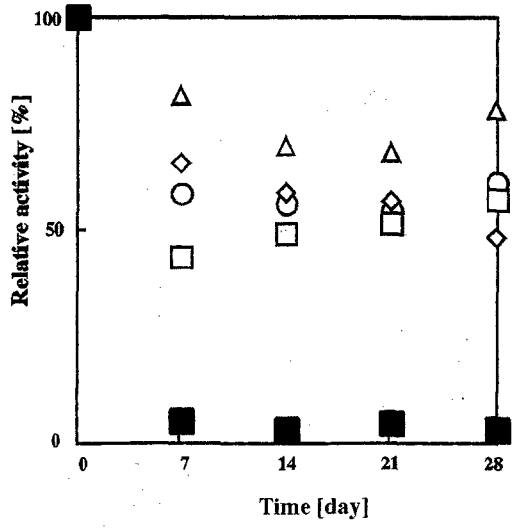

Fig. 3 Time dependence of enzymatic activity of native (■), P-PMPC (□), P-PMB5 (○), P-PMB25 (◇) and P-PNM50 (△) stored at 40 °C.

4. CONCLUSIONS

The bioconjugate polymers (PMPC, PMB) with a reactive end carboxylic group could be synthesized by photoinduced living radical polymerization. The PMPC, which is water-soluble and biocompatible polymer, maintained the secondary structure and is used as base polymer for bioconjugation. By introduction of hydrophobic unit in polymer for conjugation, its bioconjugate was maintained high enzymatic activity and stability compared to poly(MPC) conjugated papain.

5.ACKNOWLEGEMENTS

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